

## Cinnamaldehyde enhances *in vitro* parameters of immunity and reduces *in vivo* infection against avian coccidiosis

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### Abstract

The effects of cinnamaldehyde (CINN) on *in vitro* parameters of immunity and *in vivo* protection against avian coccidiosis were evaluated. *In vitro* stimulation of chicken spleen lymphocytes with CINN (25–400 ng/ml) induced greater cell proliferation compared with the medium control ( $P < 0.001$ ). CINN activated cultured macrophages to produce higher levels of NO at 1.2–5.0 µg/ml ( $P < 0.001$ ), inhibited the growth of chicken tumour cells at 0.6–2.5 µg/ml ( $P < 0.001$ ) and reduced the viability of *Eimeria tenella* parasites at 10 and 100 µg/ml ( $P < 0.05$  and  $P < 0.001$ , respectively), compared with media controls. In chickens fed a diet supplemented with CINN at 14.4 mg/kg, the levels of IL-1β, IL-6, IL-15 and interferon-γ transcripts in intestinal lymphocytes were 2- to 47-fold higher ( $P < 0.001$ ) compared with chickens given a non-supplemented diet. To determine the effect of CINN diets on avian coccidiosis, chickens were fed diets supplemented with CINN at 14.4 mg/kg (*E. maxima* or *E. tenella*) or 125 mg/kg (*E. acervulina*) from hatch for 24 d, and orally infected with  $2.0 \times 10^4$  sporulated oocysts at age 14 d. CINN-fed chickens showed 16.5 and 41.6% increased body-weight gains between 0–9 d post-infection (DPI) with *E. acervulina* or *E. maxima*, reduced *E. acervulina* oocyst shedding between 5–9 DPI and increased *E. tenella*-stimulated parasite antibody responses at 9 DPI compared with controls.

**Key words:** Cinnamaldehyde: Chickens: Coccidiosis: Immunity: Cytokines

Coccidiosis, an intestinal disease caused by several species of *Eimeria* protozoa, is an economically important disease for commercial poultry production<sup>(1)</sup>. Widespread use of antibiotic-based growth promoters has improved the efficiency of worldwide poultry production. However, due to the emergence of drug-resistant pathogens and the European Union's ban on the use of antibiotics as growth promoters in feeds, interest has shifted toward the development of alternative strategies, such as dietary supplementation with phytochemicals, to control avian coccidiosis<sup>(2)</sup>. Phytochemicals are a group of natural growth promoters derived from herbs, spices or other plants. In this regard, many medicinal foods and herbal products are highly effective in enhancing host defence against microbial infections, reducing tumorigenesis and decreasing oxidative stress<sup>(3–6)</sup>. Previous studies in our laboratory have demonstrated that chickens fed a diet supplemented with phytochemicals and subsequently challenged with *Eimeria* parasites showed reduced gut lesions, enhanced body-weight gain and decreased excreta oocyst output compared

with birds fed a control diet<sup>(7,8)</sup>. Furthermore, altered expression of immune-related genes in chickens was observed after the feeding of phytochemicals, supporting their well-known medicinal effects<sup>(9,10)</sup>. Therefore, it has been proposed that phytochemicals augment host immunity against infectious agents through their ability to alter gene expression<sup>(7,8)</sup>.

Cinnamaldehyde (CINN) is a constituent of cinnamon (*Cinnamomum cassia* Presl (Lauraceae)) that is widely used as a flavoring compound and has been traditionally used to treat human diseases, including dyspepsia, gastritis and inflammatory diseases. CINN has been reported to possess antioxidant, antimicrobial and larvicidal activities<sup>(11–13)</sup>, as well as to modulate T cell differentiation<sup>(14)</sup>. CINN has been found to be active against human liver, lung and leukaemia cancer cells in anticancer studies<sup>(15–17)</sup>, is the most potent antiproliferative constituent of *C. cassia*<sup>(18)</sup>, and its antitumour effects have also been described using a murine A375 model of human melanoma<sup>(19)</sup>.

**Abbreviations:** CINN, cinnamaldehyde; EtMIC2, purified recombinant microneme protein from *Eimeria tenella*; IFN, interferon.

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At the physiological level, CINN protects the intestinal microvilli, which are responsible for the absorption of nutrients<sup>(20–22)</sup>. Dietary feeding of CINN along with carvacrol and capsaicin, or capicum, improved feed conversion, but did not improve body-weight gain compared with that of control chickens<sup>(23–25)</sup>. While the mechanisms that are responsible for these phenomena are unknown, it has been suggested that they may involve morphological modification of gastrointestinal mucosal cells<sup>(9)</sup> and/or altered expression of metabolism-related genes<sup>(26)</sup>. Chickens fed a diet supplemented with CINN also displayed reduced intestinal colonisation by *Escherichia coli*, *Clostridium perfringens* and fungi, and increased colonisation by *Lactobacillus* spp., compared with controls<sup>(23)</sup>. Our previous microarray study showed that feeding of CINN to chickens altered the expression of sixty-two genes (thirty-one up-regulated, thirty-one down-regulated) in intestinal intra-epithelial lymphocytes<sup>(26)</sup>. Therefore, the present investigation was performed to evaluate the effects of CINN on *in vitro* parameters of immunity and to assess its ability to reduce infection against avian coccidiosis *in vivo*.

## Methods

### Spleen lymphocyte proliferation

All experiments were approved by the Agricultural Research Service Institutional Animal Care and Use Committee. Specific pathogen-free Ross/Ross broiler chickens, aged 3 weeks (Longenecker's Hatchery, Elizabethtown, PA, USA), were euthanised by cervical dislocation. Spleens were then removed and placed in Petri dishes with 10 ml of Hanks' balanced salt solution supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) (Sigma, St Louis, MO, USA). Cell suspensions were prepared by gently flushing through a cell strainer and lymphocytes were purified by density gradient centrifugation through Histopaque-1077 (Sigma)<sup>(4)</sup>. The cells were adjusted to  $1.0 \times 10^7$  cells/ml in Roswell Park Memorial Institute (RPMI) 1640 medium without phenol red (Sigma) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml), and 100 µl/well were added to ninety-six-well flat-bottomed plates containing CINN at 100 µl/well (400, 100, 50 or 25 ng/ml) from Pancosma S.A. (Geneva, Switzerland), concanavalin A (500 ng/ml; Sigma) as a positive control, or medium alone as a negative control. The cells were incubated at 41°C in a humidified incubator (Forma, Marietta, OH, USA) with 5% CO<sub>2</sub> for 48 h and cell numbers were measured using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA) at 450 nm using a microplate spectrophotometer (BioRad, Hercules, CA, USA).

### Nitric oxide production by macrophages

HD11 chicken macrophages were cultured at  $1.0 \times 10^6$  cells/ml (100 µl/well) in ninety-six-well plates with CINN at 100 µl/well (1.2, 2.5 or 5.0 µg/ml), recombinant interferon

(IFN)- $\gamma$ <sup>(27)</sup> (1.0 µg/ml) as a positive control, or medium alone as a negative control at 41°C and 5% CO<sub>2</sub> for 24 h. Cell culture supernatant fractions (100 µl) were mixed with 100 µl of Griess reagent (Sigma), incubated for 15 min at room temperature, optical densities at 540 nm were measured using a microplate spectrophotometer, and nitrite concentrations were determined using a standard curve generated with known concentrations of sodium nitrite.

### Tumour cell cytotoxicity

Retrovirus-transformed chicken RP9 B cells<sup>(4)</sup> were cultured at  $1.0 \times 10^6$  cells/ml (100 µl/well) in ninety-six-well plates with CINN at 100 µl/well (0.3, 0.6, 1.2 or 2.5 µg/ml), recombinant chicken NK-lysin (1.0 µg/ml) as a positive control, or medium alone as a negative control at 41°C and 5% CO<sub>2</sub> for 48 h, and cell numbers using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) were measured at 450 nm using a microplate spectrophotometer.

### Parasite cytotoxicity

Freshly sporulated *E. tenella* oocysts were disrupted with 0.5 mm glass beads for 5–7 s and the freed sporozoites were incubated in PBS containing 0.014 M-taurodeoxycholic acid and 0.25% trypsin to release sporozoites at 41°C for 45 min. Sporozoites were filtered, washed three times with RPMI 1640 at 2100 rpm at 4°C for 10 min, incubated at  $1 \times 10^6$ /ml with 100 or 10 µg CINN per ml at 41°C for 24 h, and viability was measured by trypan blue exclusion.

### Quantification of intestinal cytokine mRNA levels

Chickens, aged 1 d (four birds per group), were housed at 29°C in Petersime brooder units and were fed *ad libitum* with a control diet (US Department of Agriculture Feed Mill, Beltsville, MD, USA) or the control diet supplemented with CINN at 14.4 mg/kg. This concentration of CINN was chosen based upon pilot studies of cytokine mRNA levels. The control diet contained 24.2% crude protein, 4.7% fat, 2.4% fibre, 1.3% linoleic acid, 1% Ca, 0.4% available P, 0.8% K, 1.5% arginine, 1.2% lysine and 0.8% methionine + cystine. At 14 d post-hatch, the birds were euthanised by cervical dislocation. Their intestinal tissues were removed, cut longitudinally and washed three times with ice-cold Hanks' balanced salt solution containing penicillin (100 U/ml) and streptomycin (100 µg/ml) (Sigma). The mucosal layer was carefully removed using a surgical scalpel and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). Then 5 µg of total RNA were treated with 1.0 U of DNase I and 1.0 µl of 10X reaction buffer (Sigma), incubated at room temperature for 15 min, 1.0 µl of stop solution was added to inactivate DNase I, and the mixture was heated at 70°C for 10 min. RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA, USA) according to the manufacturer's recommendations. Quantitative RT-PCR oligonucleotide

primers for chicken IL-1 $\beta$ , IL-6, IL-15 and IFN- $\gamma$  and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) internal control are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA using the Mx3000P system and Brilliant SYBR Green qPCR master mix (Stratagene). Standard curves were generated using log<sub>10</sub> diluted standard RNA and the levels of individual transcripts were normalised to those of GAPDH by the QGene program<sup>(28)</sup>. Each sample was analysed in triplicate. To normalise individual replicates, the logarithmic-scaled threshold cycle ( $C_t$ ) values were transformed to linear units of normalised expression before calculating means and SEM for the references and individual targets, followed by the determination of mean normalised expression using the QGene program<sup>(28)</sup>.

### Experimental animals, diets and *Eimeria* infection

The immunomodulatory effect of CINN against avian coccidiosis was evaluated in chickens infected with *E. tenella*, *E. acervulina* or *E. maxima*. Briefly, chickens aged 1 d (twenty per group) were fed with a control diet or a diet supplemented with CINN at 14.4 or 125 mg/kg (the concentrations in diets). These concentrations of CINN were chosen based upon pilot studies of the immunomodulatory effect of CINN on *Eimeria*-infected birds. At 14 d post-hatch, the birds were transferred to cages (two birds per cage) for excreta collection and were either uninfected or orally infected with  $2.0 \times 10^4$  sporulated oocysts of *E. tenella*, *E. maxima* or *E. acervulina* as described<sup>(29)</sup>. Body-weight gains were calculated between 0 and 9 d post-infection. For determination of excreta oocyst shedding, birds were placed in cages (two birds per cage, twelve per group) and the excreta samples were collected daily between 5 and 9 d post-infection and then pooled. Oocyst numbers per bird over 4 d were calculated as described<sup>(30)</sup> using a McMaster chamber according to the formula: total oocysts/bird = (oocyst count  $\times$  dilution factor  $\times$  (excreta sample volume/counting chamber volume))/2.

### Serum antibody levels

Blood was obtained by cardiac puncture (four birds per group) following euthanasia at 9 d post-infection and sera were collected by centrifugation. Diluted sera (1:100, 100  $\mu$ l/well) were added to ninety-six-well microtitre plates pre-coated with 10  $\mu$ g per well of EtMIC2, a purified recombinant microneme protein from *E. tenella*, as described<sup>(31)</sup>, incubated with agitation at room temperature for 1 h, and washed with PBS containing 0.05 % Tween 20. Bound antibody was reacted with peroxidase-conjugated rabbit anti-chicken IgG (Sigma) and 3,3',5,5'-tetramethylbenzidine substrate (Sigma), and optical density at 450 nm was determined using a microplate spectrophotometer.

### Statistical analyses

Each sample was analysed in triplicate or quadruplicate. Statistical analyses were performed using SPSS software (SPSS 15.0 for Windows; SPSS, Inc., Chicago, IL, USA), and all data were expressed as mean values with their standard errors. Comparisons of the mean values were performed by one-way ANOVA, followed by Student's *t* test or Duncan's multiple-range test, and differences were considered statistically significant at  $P < 0.05$ .

## Results

### Effects of cinnamaldehyde on in vitro and in vivo parameters of immunity

Dietary CINN increased splenocyte proliferation at all concentrations tested compared with the medium control ( $P < 0.001$ ) (Fig. 1(A)). Cell proliferation with CINN at 400 ng/ml was comparable with that of the concanavalin A-stimulated positive control. NO levels in the cell culture media of CINN-treated HD11 macrophages were greater than those of cells treated with medium alone ( $P < 0.001$ ) (Fig. 1(B)). CINN had no observable toxic effects on spleen cells or macrophages at any of the concentrations tested. Treatment of RP9 tumour

**Table 1.** Oligonucleotide primers used for quantitative RT-PCR of chicken cytokines

RNA target	Primer sequences	PCR product size (bp)	Accession no.
GAPDH			
Forward	5'-GGTGGTGCTAAGCGTGTTAT-3'	264	K01458
Reverse	5'-ACCTCTGTCATCTCTCCACA-3'		
IL-1 $\beta$			
Forward	5'-TGGGCATCAAGGGCTACA-3'	244	Y15006
Reverse	5'-TCGGGTTGGTTGGTGATG-3'		
IL-6			
Forward	5'-CAAGGTGACGGAGGAGGAC-3'	254	AJ309540
Reverse	5'-TGGCGAGGAGGATTCT-3'		
IL-15			
Forward	5'-TCTGTTCTTCTGTTCTGAGTGATG-3'	243	AF139097
Reverse	5'-AGTGATTGCTTCTGTCTTTGGTA-3'		
IFN- $\gamma$			
Forward	5'-AGCTGACGGTGGACCTATTATT-3'	259	Y07922
Reverse	5'-GGCTTTGCGCTGGATTC-3'		

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN, interferon.

cells with CINN at 0.6, 1.2 or 2.5  $\mu\text{g/ml}$  reduced cell viability compared with the medium control ( $P<0.001$ ) (Fig. 1(C)). CINN decreased *E. tenella* sporozoite viability at 10  $\mu\text{g/ml}$  ( $P<0.05$ ) and 100  $\mu\text{g/ml}$  ( $P<0.001$ ) compared with the medium control (Fig. 1(D)). Finally, the levels of transcripts encoding the pro-inflammatory cytokines IL-1 $\beta$  and IL-6, as well as the Th1-type cytokines IL-15 and IFN- $\gamma$ , were increased in the intestine of chickens fed CINN at 14.4 mg/kg by 12, 2.0, 10 and 47-fold, respectively, compared with each of the non-supplemented control groups ( $P<0.001$ ) (Fig. 2).

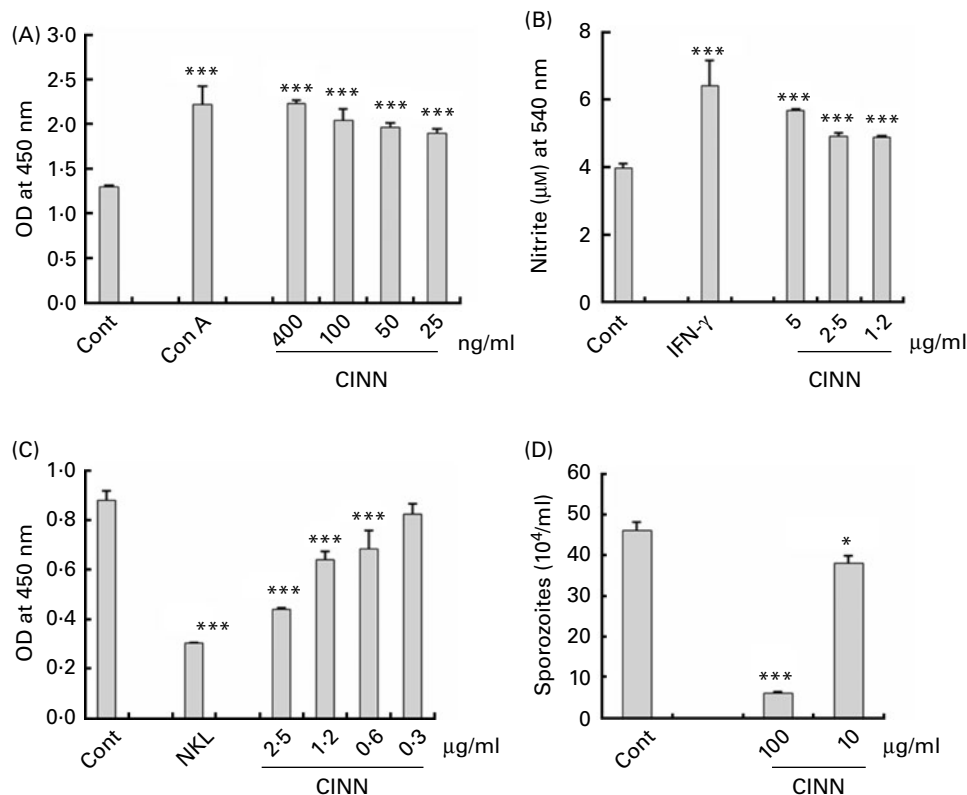
#### Effect of cinnamaldehyde on *in vivo* protection against avian coccidiosis

Chickens that were fed CINN at 125 mg/kg and infected with *E. acervulina*, or were fed CINN at 14.4 mg/kg and infected with *E. maxima*, had significantly ( $P<0.05$ ) increased body-weight gains between 0 and 9 d post-infection compared with infected birds given a non-supplemented diet (Fig. 3(A) and (B)). By contrast, feeding of CINN at 14.4 mg/kg had no effect on body-weight gain of *E. tenella*-infected animals (Fig. 3(C)).

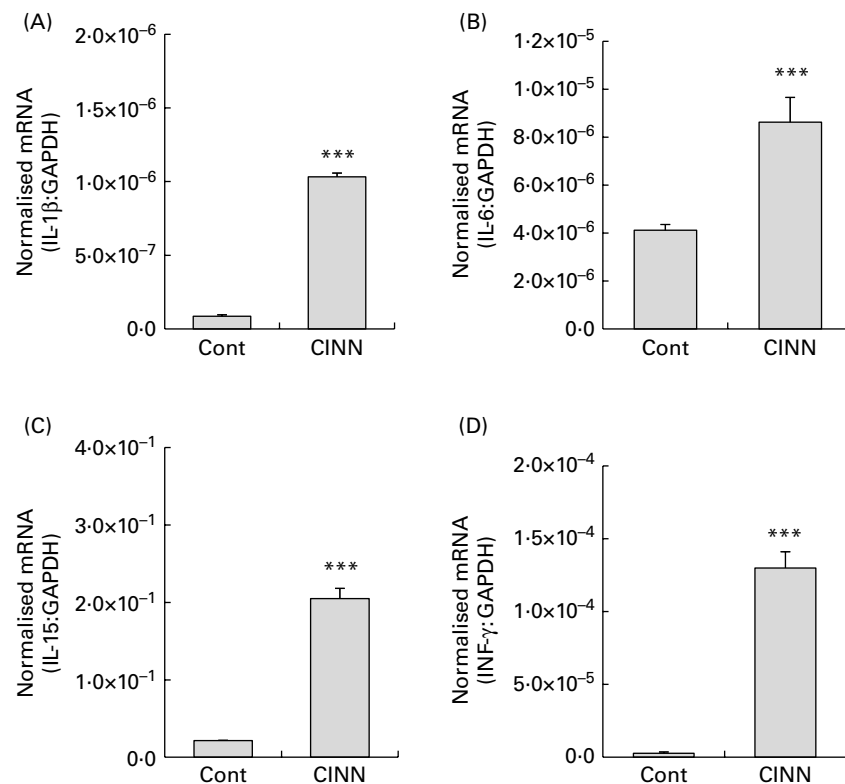
Excreta oocyst number was reduced by 41% in *E. acervulina*-infected chickens fed with CINN at 125 mg/kg compared with infected animals given the non-supplemented diet ( $P<0.01$ ) (Fig. 4). By contrast, excreta oocyst numbers of *E. maxima*- or *E. tenella*-infected chickens given non-supplemented or CINN-supplemented (14.4 mg/kg) diets were equal. Finally, the levels of serum antibodies reactive with the recombinant EtMIC2 protein were increased by 98% in *E. tenella*-infected chickens fed the CINN-supplemented diet ( $P<0.001$ ), but not in the birds given the CINN diet and infected with *E. acervulina* or *E. maxima*, when compared with animals on the control diet (Fig. 5).

#### Discussion

The present study demonstrated that CINN enhanced *in vitro* and *in vivo* parameters of immunity and reduced experimental *Eimeria* infection *in vivo*. It should be noted, however, that these effects were *Eimeria* species specific and were not detected across all observations. Treatment of chicken spleen cells or HD11 macrophages with CINN increased proliferation and NO production, respectively, and treatment of chicken RP9 tumour cells or *E. tenella* sporozoites with CINN decreased cell viability. For the *in vivo* studies, feeding



**Fig. 1.** Effects of cinnamaldehyde (CINN) treatments on *in vitro* parameters of immunity. (A) Spleen cells were treated with the indicated concentrations of CINN, concanavalin A (Con A) (500 ng/ml) or medium (control; Cont) for 48 h and viable cell numbers were measured using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (WST-8). (B) HD11 macrophages were treated with the indicated concentrations of CINN, recombinant chicken interferon (IFN- $\gamma$ ) (1.0  $\mu\text{g/ml}$ ) or medium (Cont) for 24 h and NO levels were measured using Griess reagent. (C) RP9 tumour cells were treated with the indicated concentrations of CINN, chicken NK-lysin (NKL; 1.0  $\mu\text{g/ml}$ ) or medium (Cont) for 48 h and viable cell numbers were measured using WST-8. (D) *Eimeria tenella* sporozoites were treated with the indicated concentrations of CINN or medium (Cont) for 24 h and viability was assessed by trypan blue exclusion. OD, optical density at 450 or 540 nm. Values are means ( $n$  4), with standard errors represented by vertical bars. Mean value was significantly different from that of the medium-treated (Cont) group: \*  $P<0.05$ , \*\*\*  $P<0.001$  (Student's *t* test).



**Fig. 2.** Effects of a cinnamaldehyde (CINN)-supplemented diet on intestinal cytokine transcript levels. Chickens were fed a non-supplemented diet (control; Cont) or a diet supplemented with CINN at 14.4 mg/kg. At 14 d post-hatch, intestinal tissue was removed and the levels of transcripts for IL-1 $\beta$  (A), IL-6 (B), IL-15 (C) and interferon (IFN)- $\gamma$  (D) were quantified by real-time RT-PCR. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Values are means ( $n$  12), with standard errors represented by vertical bars. \*\*\* Mean value was significantly different from that of the group fed the non-supplemented diet (Cont) ( $P$  < 0.001; Student's  $t$  test).

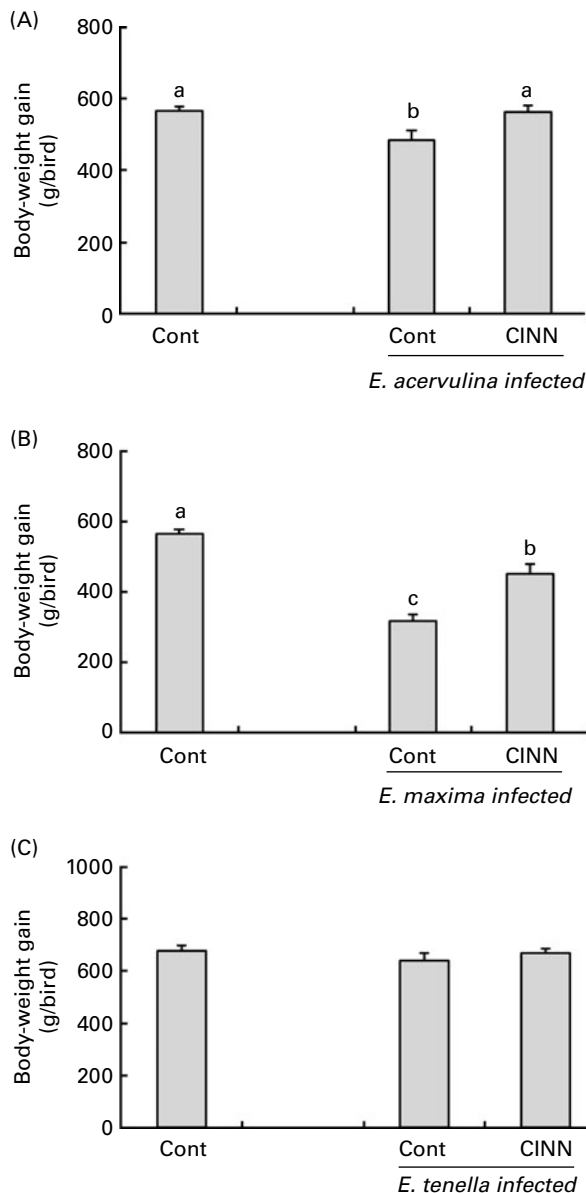
of CINN increased the levels of intestinal mRNA encoding IL-1 $\beta$ , IL-6, IL-15 and IFN- $\gamma$ , reduced *E. acervulina*- and *E. maxima*-induced body-weight loss, reduced *E. acervulina* oocyst shedding, and increased the *E. tenella*-stimulated EtMIC2 antibody response compared with feeding of the control diet.

Previous studies have demonstrated the beneficial effects of plant extracts in chicken diets for reducing the number of pathogenic gut bacteria without increasing digestibility of nutrients (crude protein, fibre and amino acids), and reducing body-weight loss due to *Eimeria* infection<sup>(23,32)</sup>. The results of the present study revealed that CINN-fed birds showed increased body-weight gain after *E. acervulina* or *E. maxima* infection and decreased oocyst shedding following *E. acervulina* infection compared with controls. The significant effect of CINN on body-weight gain and oocyst reduction in the *E. acervulina*-infected animals compared with the *E. maxima*- and *E. tenella*-infected groups may have been due to the relatively higher concentration of the phytochemical in the diet fed to *E. acervulina* birds (125 *v.* 14.4 mg/kg). However, when compared with the previous concentrations of dietary supplement used in coccidiosis control (ranging from 200 to 1000 mg/kg)<sup>(8,29,31)</sup>, the concentration used in the present study (125 mg/kg) was relatively low. In addition, given that the challenge dose of *Eimeria* parasites used in the present investigation ( $2.0 \times 10^4$  oocysts per bird) is likely to be

considerably higher than the exposure level in commercial production flocks, it remains to be determined whether the lower CINN supplementation also may provide protection against coccidiosis in poultry raised under normal field conditions. It is interesting to note, however, that chickens provided with the higher dose of CINN and infected with *E. acervulina* nevertheless failed to generate antibodies that cross-reacted with EtMIC2.

T and B lymphocytes, macrophages, monocytes and natural killer cells mediate innate and acquired immune defences. Macrophages play an important role in host defence against infectious agents and tumours, in part, through the production of effector molecules, such as NO, and IFN- $\gamma$ -stimulated NO production by chicken macrophages has been reported<sup>(33)</sup>. Previous studies have demonstrated that the effects of plant extracts on host defence against microbial pathogens and tumours directly correlated with increased cell-mediated immunity<sup>(4,5,8)</sup>. The present results demonstrating a stimulatory effect of CINN on *in vitro* NO production in chicken macrophages may be related to the result of the *in vivo* study in which CINN increased IFN- $\gamma$  expression in the intestine. Moreover, the present data correlate well with previous reports that documented the bioactive properties of medicinal foods and herbs on macrophage activation<sup>(6,34,35)</sup>. On the other hand, other studies have reported that CINN





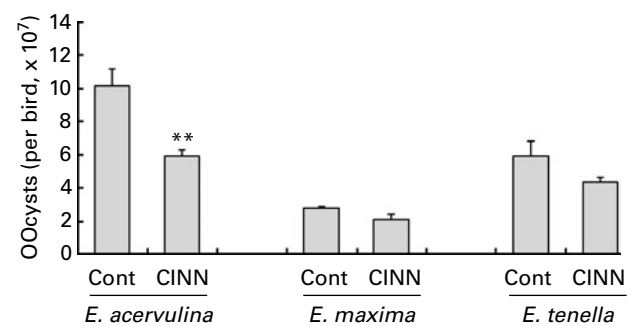
**Fig. 3.** Effect of cinnamaldehyde (CINN)-supplemented diets on body-weight gain following *Eimeria* infection. Chickens were fed a non-supplemented diet (control; Cont) or diets supplemented with CINN at 125 mg/kg (A) or 14.4 mg/kg (B, C). At 14 d post-hatch, chickens were uninfected or orally infected with  $2.0 \times 10^4$  sporulated oocysts of *Eimeria acervulina* (A), *E. maxima* (B) or *E. tenella* (C) and body-weight gains were measured between 0 and 9 d post-infection. Values are means ( $n = 20$ ), with standard errors represented by vertical bars. <sup>a,b,c</sup> Mean values with unlike letters were significantly different ( $P < 0.05$ ; Duncan's multiple-range test). The improvement in body-weight gain of birds fed the CINN-supplemented diet compared with those fed the non-supplemented diet following infection with *E. acervulina* was 16.5% (A). The improvement in body-weight gain of birds fed the CINN-supplemented diet compared with those fed the non-supplemented diet following infection with *E. maxima* was 41.6% (B).

suppressed NO production by lipopolysaccharide-activated mouse macrophages<sup>(36)</sup>.

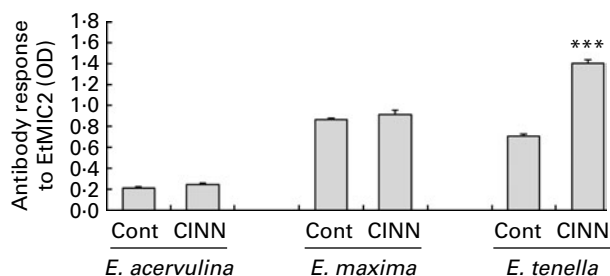
Protective immunity to *Eimeria* infection is accompanied by the production of a collection of cytokines, chemokines and other protein mediators of local inflammatory responses<sup>(1)</sup>. For example, IL-1 $\beta$  is a pro-inflammatory cytokine that is

produced by macrophages, monocytes, and dendritic cells that play a central role in the regulation of immune and inflammatory responses. In mammals, IL-1 $\beta$  increases the expression of cell adhesion molecules on endothelial cells to enable the transmigration of blood leucocytes to extravascular sites of infection<sup>(37)</sup>. In chickens, IL-1 $\beta$  given simultaneously with a DNA vaccine following oral *Eimeria* infection exerted an adjuvant effect by reducing excreted oocyst shedding<sup>(38)</sup>. IL-6 is produced by T cells and macrophages and acts as both a pro-inflammatory and an anti-inflammatory cytokine, depending upon the context of its expression, whereas IL-15 is primarily secreted by mononuclear phagocytes and enhances the activation of memory T cells<sup>(39)</sup>. Chicken IL-15 promoted the survival of T lymphocytes and natural killer cells and enhanced protective immunity to experimental coccidiosis when co-administered with a DNA vaccine<sup>(38,40)</sup>. IFN- $\gamma$  is a common marker of cellular immunity and high levels of IFN- $\gamma$  are associated with protective immune responses to coccidiosis<sup>(29)</sup>. Administration of recombinant IFN- $\gamma$  to chickens increased resistance against coccidiosis, significantly reduced the intracellular development of *Eimeria* parasites<sup>(27)</sup>, and showed an adjuvant effect when given with a DNA vaccine<sup>(41)</sup>. On the basis of these reports, we predict that enhanced production of these cytokines in birds that are continuously fed with a diet supplemented with CINN at a relatively low concentration may provide a novel opportunity to increase anti-coccidial immunity and reduce parasite fecundity.

In conclusion, the present results provide the first demonstration that, in general, dietary CINN enhances *in vitro* parameters of immunity and reduces *Eimeria* infection of chickens. While these effects were *Eimeria* species specific and were not observed across all experiments, the following key observations were reproducibly validated: dietary CINN attenuated *E. acervulina* and *E. maxima*-induced body-weight loss, decreased *E. acervulina* oocyst shedding, and increased *E. tenella*-stimulated EtMIC2 antibody responses compared with the non-supplemented control diet. Further



**Fig. 4.** Effect of cinnamaldehyde (CINN)-supplemented diets on excreta oocyst shedding following *Eimeria* infection. Chickens were fed a non-supplemented diet (control; Cont) or diets supplemented with CINN at 125 or 14.4 mg/kg. At 14 d post-hatch, chickens were orally infected with  $2.0 \times 10^4$  sporulated oocysts of *Eimeria acervulina* (125 mg CINN/kg), *E. maxima* (14.4 mg CINN/kg) or *E. tenella* (14.4 mg CINN/kg) and excreta oocyst numbers were measured between 5 and 9 d post-infection. Values are means ( $n = 12$ ), with standard errors represented by vertical bars. \*\* Mean value was significantly different from that of the group fed the non-supplemented diet (Cont) ( $P < 0.01$ ; Student's *t* test).



**Fig. 5.** Effect of cinnamaldehyde (CINN)-supplemented diets on EtMIC2 (a purified recombinant microneme protein from *Eimeria tenella*) serum antibody levels following *Eimeria* infection. Chickens were fed a non-supplemented diet (control; Cont) or diets supplemented with CINN at 125 or 14.4 mg/kg. At 14 d post-hatch, chickens were orally infected with  $2.0 \times 10^4$  sporulated oocysts of *E. acervulina* (125 mg CINN/kg), *E. maxima* (14.4 mg CINN/kg) or *E. tenella* (14.4 mg CINN/kg) and EtMIC2 serum antibody levels were measured at 9 d post-infection. OD, optical density at 450 nm. Values are means ( $n$  4), with standard errors represented by vertical bars. \*\*\*Mean value was significantly different from that of the group fed the non-supplemented diet (Cont) ( $P < 0.001$ ; Student's  $t$  test).

studies are necessary to better understand the underlying immune mechanisms that are responsible for these effects and to assess the ability of dietary CINN to provide a safe and effective alternative disease control method against avian coccidiosis in commercial production facilities.

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

There are no conflicts of interest.

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